

O P I C  
OFFICE DE LA PROPRIÉTÉ  
INTELLECTUELLE DU CANADA



C I P O  
CANADIAN INTELLECTUAL  
PROPERTY OFFICE

Ottawa, Hull K1A 0C9

|           |            |
|-----------|------------|
| (21) (A1) | 2,141,412  |
| (86)      | 1993/08/02 |
| (43)      | 1994/02/17 |

(51) Int.Cl. <sup>6</sup> C12N 15/72; C12N 1/21

**(19) (CA) APPLICATION FOR CANADIAN PATENT (12)**

(54) Cloning and/or Sequencing Vector

(72) Bernard, Philippe - Belgium ;  
Gabant, Philippe - Belgium ;

(71) Université Libre de Bruxelles - Belgium ;

(30) (BE) 92/00696 1992/07/31

(57) 17 Claims

**Notice:** This application is as filed and may therefore contain an incomplete specification.



Industry Canada Industrie Canada

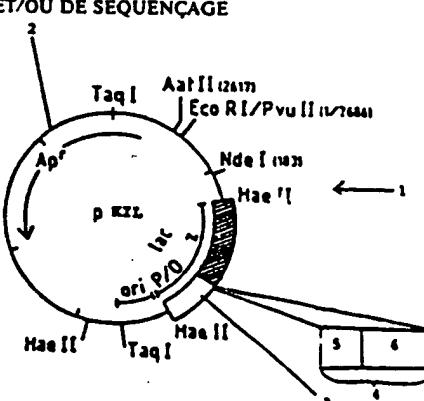
Canada

PCT

ORGANISATION MONDIALE DE LA PROPRIÉTÉ INTELLECTUELLE  
Bureau International



DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)

|  |  |   |  |
|--|--|---|--|
| <p>(51) Classification internationale des brevets <sup>5</sup> :<br/>C12N 15/64, 15/70, 1/21 //<br/>(C12N 1/21, C12R 1/19)</p>   |  | A3  | <p>(11) Numéro de publication internationale: WO 94/03616<br/>(43) Date de publication internationale: 17 février 1994 (17.02.94)<br/><b>2141412</b></p> |
| <p>(21) Numéro de la demande internationale: PCT/BE93/00051<br/>(22) Date de dépôt international: 2 août 1993 (02.08.93)</p>   |  | <p>(74) Mandataire: VAN MALDEREN, Michel; Office Van<br/>Malderen, Avenue J.-S. Bach 22/43, B-1080 Bruxelles<br/>(BE).</p>  |  |
| <p>(30) Données relatives à la priorité:<br/>92/0696 31 juillet 1992 (31.07.92) BE</p>   |  | <p>(81) Etats désignés: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP,<br/>KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO,<br/>RU, SD, SK, UA, US, VN, brevet européen (AT, BE,<br/>CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL,<br/>PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA,<br/>GN, ML, MR, NE, SN, TD, TG).</p> |  |
| <p>(71) Déposant (pour tous les Etats désignés sauf US): UNIVERSITÉ LIBRE DE BRUXELLES (BE/BE); Avenue F.D. Roosevelt 50 CP 165, B-1050 Bruxelles (BE).</p>  |  | <p>Publié<br/>Avec rapport de recherche internationale.<br/>Avant l'expiration du délai prévu pour la modification<br/>des revendications, sera republiée si de telles<br/>modifications sont requises.</p>   |  |
| <p>(72) Inventeurs: et<br/>(73) Inventeurs/Déposants (US seulement): BERNARD, Philippe (BE/BE); 52, rue Cluysenaar, B-1060 Bruxelles (BE), GABANT, Philippe (BE/BE); 9, rue Van Bemmec, B-1030 Bruxelles (BE).</p>   |  | <p>(88) Date de publication du rapport de recherche<br/>internationale: 31 mars 1994 (31.03.94)</p>   |  |
| <p>(54) Titre: CLONING AND/OR SEQUENCING VECTOR<br/>(54) Titre: VECTEUR DE CLONAGE ET/OU DE SEQUENÇAGE</p>   |  |   |  |
|   |  |   |  |
| <p>(57) Abstract</p> <p>A cloning and/or sequencing vector (1) comprises, incorporated in an autonomous replication vector (2), at least one nucleotide promoter sequence (3) and at least one nucleotide sequence (4) coding for a fusion protein active as a poison. Said nucleotide sequence (4) is obtained by fusion of a codin' nucleotide sequence (5), comprising several unique cloning sites and a nucleotide sequence (6) coding for a poison protein. The vector host cell of the invention is also disclosed.</p> |  |   |  |

(57) Abrégé

La présente invention concerne un vecteur de clonage et/ou de séquençage (1) comprenant incorporé dans un vecteur à réplication autonome (2), au moins une séquence nucléotidique promotrice (3) et au moins une séquence nucléotidique (4) codant pour une protéine de fusion active en tant que poison; ladite séquence nucléotidique (4) étant obtenue par la fusion d'une séquence nucléotidique codante (5), comprenant plusieurs sites uniques de clonage et d'une séquence nucléotidique (6) codant pour une protéine poison. La présente invention concerne également la cellule hôte du vecteur selon l'invention.

The cloning vectors of the state of the art suffer from the disadvantage of having to be maintained in a host strain which includes the LacI<sup>R</sup> repressor in episomal form, or the CI repressor, in order to inactivate the promoter and prevent expression of the killer gene, thus leading to the death of the host strain.

In addition, if it is desired to use this strain to produce a large number of copies of the cloning vectors, the repressor will not be adequate for preventing either a selective pressure which modifies the cytotoxic activity of the vector or a "genetic leakage", that is to say expression of certain copies of the vector and death of the host strain.

Consequently, none of the documents of the state of the art describes a cloning vector which can incorporate large nucleotide fragments, which is easy to manipulate and which can be produced by a microorganism on an industrial scale; that is to say, which can be produced in a large number of copies by a microorganism without bringing about the death of the latter.

#### Objects of the invention

The present invention aims to supply a novel cloning and/or sequencing vector, and also its host strain, which are simple and relatively inexpensive to construct and produce, and which enable recombinant clones to be selected directly, without suffering from the disadvantages of the abovementioned state of the art.

A particular object of the present invention is to obtain a vector which permits specific and certain selection of the recombinant clones.

Another object of the present invention is directed towards obtaining a vector which permits the sequencing, amplification and/or characterization, using the same primer, of any foreign DNA fragment (whatever its size) in the recombinant clones.

An additional object of the present invention is directed towards obtaining a vector which also permits simple extraction of this foreign DNA fragment from the recombinant clone.

2141412

WO 94/03616

- 5 -

PCT/BR93/00051

A final object of the present invention is directed towards obtaining a host strain for the said vector which allows a large number of copies of the said vector to be produced without bringing about selection 5 pressure which modifies the cytotoxic activity of the said vector or causing the death of the host strain.

Characteristic elements of the invention

The invention relates to a novel cloning and/or sequencing vector which includes, incorporated into an 10 autonomously replicating vector, at least one promoter nucleotide sequence and at least one nucleotide sequence encoding a fusion protein which is active as a poison, the said nucleotide sequence being obtained by fusing a coding nucleotide sequence which includes several unique 15 cloning sites and a nucleotide sequence which encodes a protein poison.

Preferably, the autonomously replicating vector is a recombinant virus or a recombinant plasmid such as a pUC plasmid.

20 The promoter nucleotide sequence can comprise any promoter, which permits expression of the nucleotide sequence encoding a fusion protein which is active as a poison.

25 Preferably, this promoter nucleotide sequence consists of the Lac operon promoter.

According to one preferred embodiment of the invention, the unique cloning sites (MCS) of the nucleotide sequence which is fused to the nucleotide sequence which encodes the protein poison are at ... from 30 the remainder of the nucleotide sequence of the vector according to the invention.

Advantageously, the nucleotide sequence of the gene which encodes the protein poison comprises all or 35 part of the nucleotide sequence of the wild-type gene which encodes the protein CcdB.

Preferably, the nucleotide sequence of the gene which encodes the protein poison lacks the cleavage site for the restriction enzyme SmaI.

Another aspect of the invention relates to a

2141412

WO 94/03616

- 6 -

PCT/BE93/00051

procaryotic cell which is transformed with the cloning vector according to the invention.

5 The invention also relates to a procaryotic host cell for the vector according to the invention which possesses a chromosomal  $I^r$  and an elevated transformation efficiency, and which possesses a mutation conferring resistance to the poison activity of the fusion protein, and/or which possesses a gene encoding a protein which is an antipoison to the fusion protein.

10 Preferably, the procaryotic host cell for the vector according to the invention possesses a mutation in the gene encoding subunit A, or in the gene encoding subunit B, of the gyrase, and conferring resistance to the fusion protein, and/or a gene which encodes the 15 protein CcdA which is an antipoison to the fusion protein.

20 Preferentially, the procaryotic cell is an *Escherichia coli* cell which possesses a mutation which is responsible for replacing arginine 462 with a cysteine in the amino acid sequence of the GyrA polypeptide of the gyrase, thereby conferring resistance to the fusion protein.

25 Preferably, this procaryotic host cell also possesses the  $LacI^r$  mutation.

30 The present invention also relates to fragments of the vector according to the invention, in particular primers for sequencing and/or amplifying (for example by PCR) the foreign nucleotide fragments inserted into the vector according to the invention.

35 Preferably, these primers consist of sequences of from 10 to 30 nucleotides which hybridize to nucleotide sequences which are situated on either side of the nucleotide sequence of the vector according to the invention which contains several unique cloning sites.

35 A final aspect of the invention relates to the use of the vector according to the invention for selecting and sequencing recombinant clones.

Brief description of the figures

- Figure 1 is a diagrammatic representation of a

2141412

WO 94/03616

- 7 -

PCT/BE93/00051

cloning vector according to the present invention.

- Figure 2 represents the nucleotide sequence of the gene *ccdB*, which encodes the protein *CcdB*.

5 - Figures 3 and 4 represent the nucleotide sequences which encode the fusion proteins of the cloning vectors pKILL18 and pKILL19, respectively. These sequences are provided with a nucleotide sequence which contains multiple unique cleavage sites for different restriction enzymes. These cloning vectors pKILL18 and pKILL19 were 10 obtained by *in vitro* recombination between the wild-type *ccdB* gene of the F plasmid and plasmids pUC18 and pUC19, respectively.

Description of a preferred embodiment of the invention

According to the invention, the cloning and/or sequencing vector 1 includes, incorporated into an autonomously replicating vector 2, at least one promoter nucleotide sequence 3 and at least one nucleotide sequence 4 which encodes a fusion protein which is active as a poison, the said nucleotide sequence 4 being 15 obtained by fusing a coding nucleotide sequence 5 (or polylinker) which encompasses several (multiple) unique cloning sites (MCS), and a nucleotide sequence (6) which encodes a protein poison.

20 An autonomously replicating vector 2 is understood to mean any nucleotide construct, such as a virus or a plasmid (preferably a recombinant plasmid of the PUC series), which is capable of being introduced into a microorganism, of recombining therein and/or of replicating therein.

25 Figure 1 shows a diagrammatic representation of a cloning vector according to the present invention, which vector is constructed from a plasmid of the pUC series (pUC18 and pUC19), which is described by Norranuer et al (Construction of improved M13 vectors using oligo-deoxinucleotide-directed mutagenesis, *Gene*, 26, pp. 101-106 (1983)) and by Yanisch-Perron et al (Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene*, 33, pp. 103-119 (1985)).

2141412

WO 94/03616

- 8 -

PCT/BE93/00051

A coding nucleotide sequence 5 encompassing several (multiple) unique cloning sites (MCS) is understood to mean a short coding sequence (or polylinker) which comprises several cleavage sites for restriction 5 enzymes.

The advantage of having a polylinker in the vector according to the invention is that different cloning sites are located on a single short sequence, thereby permitting:  
10 - rapid sequencing and amplification, using the same primers, of any DNA fragment which is inserted into this vector.  
- rapid extraction of the cloned fragment, facilitated by the proximity of the restriction sites. Thus, in contrast  
15 to the state of the art, this proximity avoids sequencing, amplifying and characterizing useless fragments from other sequences of the vector according to the invention.

Nucleotide sequence 6 encoding a protein poison 20 is understood to mean any (wild-type) nucleotide structure encoding a protein which displays an activity which is naturally poisonous and specific for one or more vital functions of a host cell.

A protein poison is also characterized by the 25 existence of an antidote or antipoison, such as the proteins CcdB and CcdA, the protein Kid and its antagonist Kis, the protein PemK and its antagonist PemL, the protein Doc and its antagonist Phd, the protein HoK and its antagonist Sok, and other poison molecules which 30 are, or are not, of plasmid origin.

In this case, the nucleotide sequence 6 encoding a protein poison consists of the wild-type gene CcdB, which encodes the protein CcdB (control of cell death), obtained from the ccd locus of the *F* plasmid (Figure 2).

The ccd locus of the *F* plasmid comprises the two wild-type genes ccdA and ccdB, also termed H and G, or letA and letD, which respectively encode proteins of 72 and 101 amino acids (Bax et al, Mini-*F* encoded proteins; identification of a new 10.5 kilodalton species. EMBO

2141412

WO 94/03616

- 9 -

PCT/BR93/00051

J.2, 1853-1861 (1983); Miki et al, Control of cell division by sex factor F in Escherichia coli. I. The 42.84-43.6 F segment couples cell division of the host bacteria with replication of plasmid DNA, J. Mol. Biol., 5 174, 605-625, (1984)).

In Escherichia coli, the CcdB protein of the F plasmid is a cytotoxin whose lethal activity is counteracted by the protein CcdA (Karoui et al, Ham22, a mini-F mutation which is lethal to host cell and promotes recA-dependent induction of lambda prophage. EMBO J.2, 1863-1868 (1983); Ogura and Hiraga Mini-F plasmid gene that couple host cell division to plasmid proliferation, Proc. Natl. Acad. Sci. USA, 80, 4784-4788 (1983); Miki et al, Control of cell division by sex factor F in Escherichia coli. Identification of genes for inhibitor protein and trigger protein on the 42.84-43.6F segment, J. Mol. Biol. 10 174, 627-646 (1984b)).

15

The molecular mechanism by which protein CcdB exerts its lethal activity has been elucidated; protein CcdB is poisonous to DNA topoisomerase II.

The type II DNA topoisomerases are essential and ubiquitous enzymes which alter the topology of the DNA by transiently introducing a double-stranded break into the DNA. During the stage of break-religation, topoisomerase 20 II forms an intermediate complex with its DNA substrate in which the enzyme is attached covalently to the 5' end of the cleaved DNA. This transitory intermediate, in which topoisomerase II is linked covalently to the DNA, has been termed the "cleavable complex" (Wang, DNA 25 topoisomerases. Annu. Rev. Biochem. 54, 665-97, 1985; Maxwell & Gellert, Mechanistic aspects of DNA 30 topoisomerases. Advan. Protein Chem. 38, 69-107, 1986; Liu, DNA topoisomerase poisons as antitumor drugs, Annu. Rev. Biochem. 58, 351-375, 1989).

35 Both in eucaryotes and in prokaryotes, the cleavable topoisomerase II-DNA complex is the target of powerful therapeutic agents, including the antibiotics of the "quinolone" family, which act on the gyrase (bacterial topoisomerase II), and anticancer agents

2141412

WO 94/03616

- 10 -

PCT/BE93/00051

(acridines and epipodophyllotoxins), which act on the mammalian topoisomerase II. The therapeutic efficacy of the topoisomerase poisons is correlated with their ability to stabilize the cleavable complex.

5 DNA topoisomerase II is an essential enzyme in all living entities and is very conserved in the evolution of the species. The CcdB protein thus displays an activity which is potentially cytotoxic for a wide variety of prokaryotic species.

10 The small size of the wild-type ccdB gene allows it to be inserted into plasmids without increasing their size excessively and consequently allows large fragments of foreign DNA to be included therein. Furthermore, given its small size, the wild-type ccdB gene of the F plasmid 15 contains very few restriction sites; it is, therefore, simpler to preserve the uniqueness of the multiple cloning sites (MCS; which are added to it).

15 Unexpectedly, the inventors observed that the in-phase fusion of the nucleotide sequence 6, encoding protein CcdB, with the coding nucleotide sequence (polylinker 5), comprising several (multiple) unique cloning sites (MCS), gave a nucleotide sequence 4 which encodes a fusion protein which is active as a poison and which makes it possible, as a consequence, to produce 20 vectors for the direct selection of recombinant plasmids 25 (killer selection).

25 The plasmids which have been obtained allow doubly digested restriction fragments to be cloned in both orientations with respect to the lac promoter. 30 Insertion of a restriction fragment into one of the unique cloning sites interrupts the genetic information of the gene fusion, leading to the synthesis of a gene fusion product which is not functional. Insertional inactivation of the gene fusion ought always to take 35 place when a termination codon is introduced or when a change is made in the reading frame.

The cells which harbor an intact cloning vector of this nature produce a poisonous fusion protein which is functional, and die.

2141412

WO 94/03616

- 11 -

PCT/BK93/00051

Insertion of a foreign DNA fragment into one of the unique cloning sites of the gene fusion interferes with production of the poison.

5 The cells which harbor a recombinant vector will be viable while cells which harbor an intact vector will not be viable. This killer selection, by simple culture on a solid medium, makes it possible to eliminate cells which harbor a non-recombinant vector (non-viable clones) and to select recombinant clones (viable clones).

10 Example I: Construction of the plasmid pKIL19

The *ccdB* gene was amplified by PCR using, as DNA template, the plasmid pULB2208 (Bernard and Couturier. The 41 carboxy-terminal residues of the *minIF* plasmid *CcdA* protein are sufficient to antagonize the killer 15 activity of the *CcdB* protein, Mol. Gen. Genet. 226, 297-304 (1991) as well as synthetic oligonucleotides.

20 The synthetic oligonucleotide sequences were selected in such a way as to create an EcoRI restriction site on either side of the wild-type *ccdB* gene in order to be able to reclone this gene in frame with the codons 25 of the MCS19 multiple cloning site and to eliminate the initiation codon of the native *ccdB* gene. The DNA resulting from the PCR reaction was digested with the enzyme EcoRI and cloned into the EcoRI site of the plasmid pUC19. The resulting plasmid, in which the EcoRI fragment was integrated in the orientation which permitted the *ccdB* gene, provided with the additional codons corresponding to the MCS19 multiple cloning sites, to be read 30 from the Lac promoter, was termed pKIL2. Plasmid pKIL2 is lethal for a wild-type bacterium (*CcdB*<sup>s</sup> sensitive).

35 pKIL2 also possesses two *SmaI* sites, one in the multiple cloning sites and the other in the central region of the *ccdB* gene. The latter was eliminated by site-directed mutagenesis. The resulting plasmid pKIL3, having a unique *SmaI* site, still has two EcoRI sites. The EcoRI site downstream of the *ccdB* gene was eliminated by filling in its cohesive ends.

40 The resulting plasmid, pKIL19 (Figure 3), thus possesses a unique EcoRI restriction site within sequence

2141412

WO 94/03616

- 12 -

PCT/BR93/00051

5, which encompasses the multiple cloning site.

Example II : construction of the plasmid pKIL18

The *ccdB* gene was amplified by PCR using, as DNA template, plasmid pKIL19 as well as synthetic oligonucleotides. The sequences of the synthetic oligonucleotides were selected in such a way as to create a HindIII site on either side of the *ccdB* gene in order to be able to reclone this gene in frame with the codons of the MCS18 multiple cloning sites. The DNA resulting from the PCR reaction was digested by the enzyme HindIII and cloned into the HindIII site of the plasmid pUC18. The resulting plasmid, in which the HindIII fragment was integrated in the orientation which permitted the *ccdB* gene, provided with the additional codons corresponding to the MCS18 multiple cloning sites, to be read from the Lac promoter, was termed pKIL4. Plasmid pKIL4 is lethal for a *CcdB*<sup>R</sup>-sensitive bacterium.

The HindIII site downstream of the *ccdB* gene was eliminated by filling in its cohesive ends. The resultant plasmid, pKIL18 (Figure 4), possesses a unique HindIII restriction site as well as a unique SmaI site (since constructed from pKIL19).

Example III : Construction of the strains *CcdB*<sup>R</sup> and *CcdB*<sup>R</sup>

In order to be able to maintain plasmids pKIL18 and pKIL19 within a bacterium, the latter has to be resistant to the lethal effect of the fusion protein which is active as a poison. Unexpectedly, the chromosomal mutation *gyrA*462 confers on the strains total resistance to the poisonous effect of the fusion protein.

Moreover, since plasmids pKIL18 and pKIL19 derive directly from plasmids pUC18 and pUC19 and express the *ccdB* genes from the Lac promoter, it is preferable to maintain these plasmids in a *LacI*<sup>R</sup> strain. Thus, while, in our case, continuous overexpression of these genes does not exert a selection pressure in favor of certain mutations, the *LacI*<sup>R</sup> strain allows expression from the Lac promoter to be reduced and conserves the bacterial machinery, thereby guaranteeing a rapid generation time (increased production of the vector by the strain).

2141412

WO 94/03616

- 13 -

PCT/BE93/00051

The strain D1210 (Sadler et al Gene 8, pp. 279-300 (1980)), derived from the strain HB101 LacI<sup>r</sup>, LacY (Maniatis et al Molecular Cloning Laboratories Man. Cold Spring Harbor Laboratory N.Y.), and characterized by a 5 chromosomal I<sup>r</sup> and increased transformation efficiency, was transformed with the plasmid pCOS2.1. This plasmid, which confers resistance to kanamycin, carries the recA gene from *Erwinia chrysanthemi* 3665 and allows recombination in *E. coli*. A lysate of P1 phage was 10 prepared on a CcdB<sup>r</sup> gyrA462, zei298::Tn10 strain and used to infect the strain D1210/pCOS2.1. The transductants which were resistant to tetracycline were selected and tested for their resistance or sensitivity to the CcdB protein. One of the CcdB<sup>r</sup> transductants was then cured of 15 plasmid pCOS2.1 and termed KIB22.

Strain KIB22 constitutes an ideal host strain for plasmids pKIL18 and pKIL19 while strain D1210 constitutes the ideal host for selecting recombinant plasmids.

Thus, strain KIB22 advantageously possesses an 20 elevated efficiency of DNA extraction (comparable to the yield of the pUC plasmids) and, unexpectedly, resistance to the fusion protein which is encoded by pKIL18 and pKIL19.

Consequently, it is possible to use this micro- 25 organism to produce the cloning vector according to the invention on an industrial scale in numerous copies without causing the death of the said microorganism.

The selection is carried out simply by spreading 30 the bacteria on a medium containing IPTG (Isopropyl-Beta-D-thiogalactopyranoside) as well as ampicillin.

Strain KIB22 was deposited with the Laboratorium voor Microbiologie-Bacteriënverzameling (LMG) [Microbiological Laboratory - Bacterial Collection] of the Belgian Coordinated Collections of Microorganisms 35 (BCCM) under No. LMG P-12601.

The cloning vector pKIL19 was deposited with the Laboratorium voor Moleculaire Biologie-Plasmiden Collectie (LMBP) [Molecular Biological Laboratory - Plasmid Collection] of the Belgian Coordinated

2141412

WO 94/03616

- 14 -

PCT/BR93/00051

Collections of Microorganisms (BCCM) under the No. LMBP  
2781.

These depositions were made in accordance with  
the provisions of the Budapest Treaty regarding the  
5 International Recognition of the Deposition of Microor-  
ganisms.

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: UNIVERSITE LIBRE DE BRUXELLES
- (B) STREET: 50 av. F. Roosevelt
- (C) CITY: Brussels
- (D) COUNTRY: BELGIUM
- (F) POSTAL CODE (ZIP): 1050
- (G) TELEPHONE: 32 2 650.31.00
- (H) TELEFAX: 32 2 650.35.12

(ii) TITLE OF INVENTION: Cloning vector

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 306 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: ccdB gene of plasmid F

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

|  |     |
|--|-----|
| ATOCAGTTTA AGGTTTACAC CTATAAAGA GAGAGCCGTT ATCGTCTGTT TGTGATGATA | 60  |
| CAGAGCTATA TTATTGACAC GCGCGGGCA CGGATGGTGA TCCCGCTGGC CAGTACACGT | 120 |
| CTGCTGTCA ATAAAGTCTC CGCTGAACTT TACCCGCTGG TGCATATCGG CGATGAAAGC | 180 |
| TOGCCGATGA TGACCCACCA TATGGGAGT GTGCCGCTCT CGTTTATCGG CGAAGGAGTG | 240 |
| GCTGATCTCA GCGACCCGCA AAATGACATC AAAACGCCA TTACCTGAT GTTCTGGGA   | 300 |
| ATATAA   | 306 |

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 420 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

2141412

(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: ccdB gene of pKIL 18

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Miki,  
(B) TITLE: Mini- $\beta$  encoded proteins  
(C) JOURNAL: EMBO  
(D) VOLUME: J.2  
(F) PAGES: 1853-1861  
(G) DATE: 1983  
(K) RELEVANT RESIDUES IN SEQ ID NO: 2: FROM 1 TO 306

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Bernard, P.  
Couturier, M.  
(B) TITLE: The 41 carboxy-terminal residues of the mini $\beta$  plasmid CcdA protein are sufficient to antagonize the killer activity of the CcdB protein  
(C) JOURNAL: Mol. Gen. Genet.  
(D) VOLUME: 226  
(F) PAGES: 297-304  
(G) DATE: 1991  
(K) RELEVANT RESIDUES IN SEQ ID NO: 2: FROM 1 TO 306

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

|  |     |
|--|-----|
| ATGACCATGA TTACGAATTG GAGCTCGTAA CCCGGGGATC CTCTAGAGTC GACCTGCAGG  | 60  |
| CATGCAAGCT TGTCTTTGCA GTTTAAGGTT TACACCTATA AAAGAGAGAG CGCTTATGGT  | 120 |
| CTGTTTGTCG ATGTACAGAG TGATATTATT GACACGCCCG GGGGACGGAT GGTGATCCCC  | 180 |
| CTGGCCAGTG CACGTCTGCT GTCAGATAAA GTCTCCCGTG AACTTTACCC GGTGGTGCAT  | 240 |
| ATCGGGGATG AAAGCTGGCG CATGATGACC ACCGATATGG CCAGTGTGCC GGTCTCCGTT  | 300 |
| ATCGGGGAAG AAGTGGCTGA TCTCAGCCAC CGCGAAAATG ACATCAAAAA CGCCATTAAAC | 360 |
| CTTATGTTCT GGGGAATATA AATGTCAGGC TCCGTTATAC ACAAGCTAGC TTGGCACTGG  | 420 |

(2) INFORMATION FOR SEQ ID NO: 3:

(1) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 416 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

2141412

(A) ORGANISM: ccdB gene of pKIL 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

|   |     |
|---|-----|
| ATGACCATGA TTACGCCAAG CTTCATGCC TGCAGGTCGA CTCTAGAGGA TCCCCGGTA   | 60  |
| CCGAGCTCGA ATTCAATTGCA GTTTAAGGT TACACCTATA AAAGAGAGAG CGCTTATCGT | 120 |
| CTGTTTGTGG ATGTACAGAG TGATATT TT GACACGCCGG GGGGACGGAT GGTGATCCCC | 180 |
| CTGGCCAGTG CACGTCTGCT GTCAAGATAAA GTCTCCCGTG AACTTTACCC GGTGGTGCA | 240 |
| ATCGGGGATG AAAGCTGGCG CATGATGACC ACCGATATGG CCAGTGTGCC GGTCTCCGTT | 300 |
| ATCGGGGAAG AAGTGGCTGA TCTCAGCCAC CGCGAAAATG ACATCAAAAC CGCCATTAAC | 360 |
| CTGATGTTCT GGGGAATATA AATGTCAGGC TCCGTTATAC ACGAATTAAAT TCAGTG    | 416 |

2141412

WO 94/03616

PCT/BK93/00051

CLAIMS

1. Cloning and/or sequencing vector which is characterized in that it includes, incorporated into an autonomously replicating vector (2), at least one promoter 5 nucleotide sequence (3) and at least one nucleotide sequence (4) which encodes a fusion protein which is active as a poison, the said nucleotide sequence (4) being obtained by fusing a coding nucleotide sequence (5), which comprises several cloning sites, and a 10 nucleotide sequence (6) which encodes a protein poison.
2. Vector according to Claim 1, characterized in that the autonomously replicating vector (2) is a recombinant virus.
3. Vector according to Claim 1, characterized in 15 that the autonomously replicating vector (2) is a recombinant plasmid.
4. Vector according to Claim 3, characterized in that the autonomously replicating vector (2) is a recombinant pUC plasmid.
5. Vector according to any one of the preceding 20 claims, characterized in that the promoter nucleotide sequence (3) consists of the Lac operon promoter.
6. Vector according to any one of the preceding 25 claims, characterized in that the unique cloning sites of the nucleotide sequence (5), which is fused to the nucleotide sequence (6) which encodes the protein poison, are absent on the remainder of the nucleotide sequence of the cloning vector.
7. Vector according to any one of the preceding 30 claims, characterized in that the nucleotide sequence (6) which encodes a protein poison comprises all or part of the nucleotide sequence of the wild-type gene which encodes the protein CcdB.
8. Vector according to Claim 7, characterized in 35 that the nucleotide sequence (6) which encodes the protein poison lacks the cleavage site for the restriction enzyme SmaI.
9. Vector according to any one of the preceding

6141412

WO 94/03616

PCT/BE93/00051

claims, characterized in that it corresponds to deposition No. LMBP2781.

10. Prokaryotic cell which is transformed with the cloning vector according to any one of the preceding 5 claims.

11. Prokaryotic host cell for the cloning vector according to any one of the preceding Claims 1 to 9, characterized in that it preferably possesses a chromosomal  $\lambda^r$  and an increased transformation efficiency 10 and possesses a mutation which confers resistance to the poisonous activity of the fusion protein and/or possesses a gene which encodes a protein which is an antipoison to the fusion protein.

12. Prokaryotic host cell for the cloning vector 15 according to Claim 11, characterized in that it possesses a mutation in the gene encoding subunit A or in the gene encoding subunit B of the gyrase, conferring resistance to the fusion protein, and/or possesses a gene which encodes the protein CcrA which is the antipoison to the 20 fusion protein.

13. Escherichia coli cell according to Claim 11 or 12, characterized in that it possesses a mutation which is responsible for replacing arginine 462 with a cysteine in the amino acid sequence of the GyrA polypeptide of the 25 gyrase, thereby conferring resistance to the fusion protein.

14. Cell according to any one of Claims 11 to 13, characterized in that it possesses the LacI $^r$  mutation.

15. Cell according to any one of the preceding Claims 30 11 to 14, characterized in that it is deposited under No. LMG-12601.

16. Fragments of the vector according to any one of Claims 1 to 9, characterized in that they consist of sequences of from 10 to 30 nucleotides which hybridize to 35 sequences situated on either side of the nucleotide sequence (5) which comprises several unique cloning sites.

17. Use of the cloning vector according to any one of Claims 1 to 9 for selecting recombinant clones.

2141412

WO 94/03616

PCT/BR93/00051

1/4

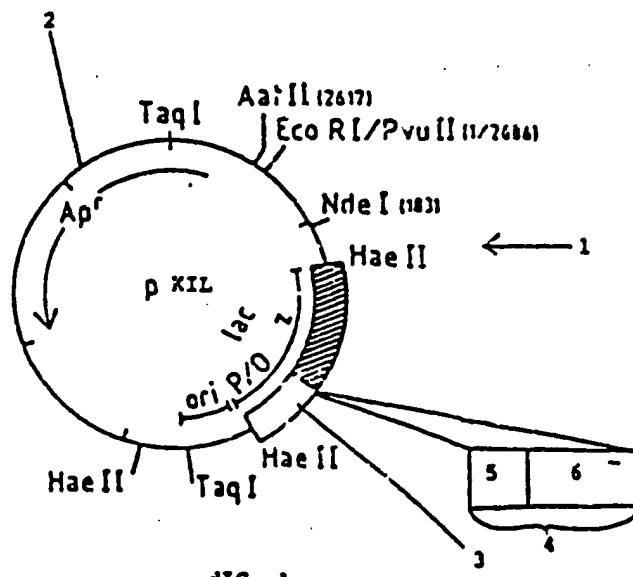


FIG. 1